STUDIES ON PTOTEASES OF PATHOGENIC BACTERIA WITH SPECIAL EMPHASIS ON ELASTASE AND NATURAL ANTI ELASTASE FACTORS

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STUDIES ON PROTEASES OF PATHOGENIC BACTERIA WITH SPECIAL EMPHASIS ON ELASTASE AND NATURAL ANTI ELASTASE FACTORS:

INTRODUCTION

Infectious diseases and bacterial resistance are major health concerns and cause of death the world over. There is a continuous emergence of new infectious diseases and re-emergence of deadly infectious diseases. The pathogenicity of the microorganisms is the cardinal cause for infection. Infection is distinguished from disease, a morbid process that does not necessarily involve microorganisms. Resistance to bacterial infection is enhanced by phagocytic cells and an intact immune system. Bacterial infectivity results from a disturbance in the balance between bacterial virulence and host resistance. The objective of the invading bacteria is to multiply in the host cell rather than to killing it or to cause a infectious disease. There are numerous physical and chemical attributes that protect the host cells from bacterial infections. These defenses include the antibacterial factors in secretion covering the mucosal surfaces and rapid rate of replacements of mucosal epithelium and skin. Of the several antibacterial factors many are peptides and these (antibacterial) peptides are classified as either non-ribosomally synthesized peptides or ribosomally synthesized peptides (RAMPS). Non ribosomolly synthesized peptides are found in bacteria and fungi. Ribosomally synthesized peptides are derived from a diverse range of species, prokaryote to humans. Once the host barrier is broken, the bacteria enter in to an environment needed for growth. The success of a bacteria to invade the host cell depends on its ability to beat the host defence mechanisms. These intrinsic characteristics of the bacteria which are encoded on the chromosome plasmid,

transposons or temperate bacteriophage DNA, determine the attacking power of the bacteria.

These characteristics are commonly referred to as the virulence factors of the infecting organisms which are also called as "Pestiferousness". Virulence is a measure of the pathogenity of an organism. The degree of virulence is related directly to the ability of the organism to cause disease despite host resistance mechanism and is dependent on number of infecting bacteria, route of entry into the body of tissue / cells, specific and non specific host defense mechanisms and most importantly the virulence factors of the bacterium. The capacity of a bacterium to cause a disease reflects on its relative pathogenicity. The pathogencity is dependent on the virulence of the bacteria. In certain infections eg. Tuberculosis the pathogenicity is host mediated and arises due to the tissue damage caused by the toxic mediators released by the lymphoid cells itself rather than bacterial toxins. Thus pathogens may have varied reasons that could be due to factors from bacteria, toxin of the host tissue or both. However, for all these sequences to happen the bacteria has to invade the host cell for which virulence play a central role.

The virulence factors are those factors produced by the micro organisms and play a cardinal role in the generation of infectious disease. These factors help bacteria to invade the host, cause disease and evade the host defense mechanism. The following are the types of virulence factors; adherence factor, invasion factors, capsules, endotoxins, exotoxins and siderophes. Of the proteinaceous toxins (exotoxins), quite a few of them are enzymes produced and delivered to host cells generally by two different methods (1)

secretion in to the surrounding milieu or (2) direct injection into the host cell cytoplasm via type III secretion systems or other mechanisms. Bacterial exotoxins can be roughly categorized into four major types based upon their amino acid composition and function (1) A-B Toxins (2) proteolytic toxins (3) pore forming toxins and (4) others. The proteolytic toxins break down specific host proteins leading to some of the characteristic clinical manifestations of the disease. Examples of proteolytic toxins include botulinum from clostridium botulinum, tetanus from clostridium tetani, elastase and protease IV from P.aeruginosa. The targets for the botulinum and tetanus toxins are synaptobrevins which prevent release of neurotransmitters. However, these toxins differs in their site of infections. Elastase and protease IV of Pseudomana aeruginosa break down cellular matrix protein allowing spread of the infection. Elastase has been considered as an important virulence factor in several bacterial species. Elastase degrades extra cellular molecules and aids tissue invasion associated with keratitis, burn tissue necrosis and cystic fibrosis.

Bacterial pathogenicity as described above, depends upon several factors which vary widely in their chemical nature and on the mode of action on the host cells. This enables the organism to counter act several of the defense mechanisms of the host cell. These factors grouped as putative virulence factors had been a topic of extensive study and development as the development of resistance in microbes are a potential threat to humans.

The bacterial resistance which could be the resultant of mutation and expression of newer proteins or other molecules, might have a better attacking ability on host cells.

Several of the studies in this direction have been on certain enzymes produced by the micro organisms particularly the proteolytic enzymes.

JUSTIFICATION AND SCOPE OF THE STUDY

There have been extensive studies carried out on the functional roles of the proteolytic enzymes produced by the microorganisms. Majority of the work on these proteolytic enzymes have been confined to elastase. Elastase enzyme has been known to break down cellular matrix protein, disrupt the structural integrity and facilitate invasion of tissues by pathogenic microorganisms.(1)

There have been reports on the ability of antiprotease substances in human sera capable of inhibiting microbial proteases and which are thought to protect the cell against proteolytic attack. In comparison, human neutrophil elastase (HNE) which is released during the attack of the invading microorganism has been shown to be inhibited by the anti proteolytic substances produced by microorganism which would combat the destructive nature of HNE on the microorganism, and this in turn would benefit the microorganism to facilitate the invasive process. Activated neutrophils release oxygen radicals and proteolytic enzymes to destroy bacteria which further damage the epithelium and protective proteinase inhibitor. HNE is stored principally in the neutrophils and is released when neutrophils encounter foreign pathogens or antigens in blood to degrade them so that the body is protected from harmful factors. (2). However, uncontrolled secretion of elastase may trigger off destructive processes associated with various chronic diseases such as rheumatoid arthritis, emphysema and psoriasis (3).

Thus, both these factors when taken into consideration play an important role either in the establishment of an infectious disease or the prevention of it. Hence, the significance of studying proteolytic enzymes further with special reference to elastase is still meaningful as there are many pathogenic organisms producing these enzymes and acting as putative virulent factors.

The present study is designed by systematically studying the microorganisms belonging to both pathogenic and non pathogenic strains on their ability to produce proteolytic enzymes. Further, the proteolytic actively exhibited by these organisms would be categorized according to the substrate specificity.

This study also aims to purify and characterize the elastase enzymes from the pathogenic and non pathogenic organisms in order to, compare the kinetics of these two enzymes, as kinetic studies might provide an insight into the activity of the enzymes produced by these pathogenic and non pathogenic organisms. Studies would be further extrapolated to compare the purified enzymes with HNE. The action of human sera anti proteases would also be examined. Though, there are a good amount of studies available in literature, on some of the aspects mentioned above, not much data is available on the nature of anti elastase substances which could be present in medicinal plants. The use of plant extracts for wound healing, treatment of skin lesions etc. is widely practiced. So characterization of anti elastase factors present in medicinal plants and their action on microbial elastase might explain and justify the use and anti bacterial properties these agents.

In addition comparison of the kinetic characteristics of the enzyme in the pathogenic and non pathogenic strains may explain the pathogenicity / virulence exhibited by disease causing microorganisms which is not well documented in literature.

REVIEW OF LITERATURE:

In 1890 Robert Koch postulated guidelines to establish a standard for evidence of causation in infectious disease. His postulates became the gold standard to define microbial virulence for over 100 years, despite limitations to their experimental applications for a number of microorganisms. Revisions of Koch's postulates were introduced to encompass those limitations in which immunological and/or epidemiological proof of causation was added (4). With the development of molecular biological techniques, it became possible to identify the genes encoding those factors responsible for virulence. This resulted in molecular microbiology, in which the role and function of specific genes and the factors they encode in bacterial virulence was the subject of investigations.

Bacterial pathogens infect a wide variety of evolutionary distinct hosts, including both lower and higher eukaryotes. In all of these cases, the pathogen must have the ability to recognize, become associated with, exploit the nutrient reserves of, and combat the defense responses of its specific host. To accomplish these tasks, pathogens use an extensive arsenal of virulence-related factors. (5)

Many pathogens cause disease in a single or limited number of host species as a consequence of a long coevolutionary history. However, the interactions between host and pathogens that limit host range and determine host resistance or susceptibility are poorly understood. Although many bacterial virulence components are thought to be host-specific, numerous studies have demonstrated the existence of what appear to be

universal virulence mechanisms used by diverse bacterial species (6). Similarly, recent work has revealed common features underlying host defense responses against pathogens in plants, insects and mammals (7). Thus, some of the underlying virulence mechanisms of pathogens, as well as the host defenses against them, are likely to have ancient evolutionary origins preserved across phylogeny.

Several bacterial pathogens have evolved to survive and replicate within the host cells after invasion. In animals, the host cell types in which pathogen can survive include non phagocytic cells such as epithelial and endothelium and professional phagocytes such as macrophages and neutrophils. The ability to survive and replicate inside of phagocytic cells is remarkable since these cells possess mechanism to destroy the ingested bacteria. These killing mechanism include the production of reactive oxidative intermediates the lowering of pH of bacteria containing vacuoles and the activation of degradtive proteases. The strategies / mechanism that bacteria use to avoid killing via these have been studied well (6).

The success of a microbe during pathogenesis relies greatly in its ability to serve and respond to the myriad of environment in the host cell. This requires the use of a repertoire of genetic functions on the part of micro organisms which are independently regulated in response to environmental signals encountered inside the infected host. The regulation and timely expression of virulence factors is very important during the natural course of infection. (8)

There has been extensive studies on virulence factors in micro organisms causing variety of diseases in the infected (9). There have been a quite good detailed knowledge about some of the extra cellular proteins considered to be virulent and pathogenic. Some of such proteins studied include proteolytic enzymes particularly the elastase. (10) The presence of the elastase enzyme has been established and documented well in cases of pathogenic organisms such as Pseudomonas, Staphylococcus, Aspergillus etc. etc., (11,12,13). The reason for elastase to be a putative virulent factor could be attributed to its potential ability to facilitate tissue invasion through the destruction of connective tissue elastin, collagen etc, in addition to its action on transferrin, Ig and on some of the components of complement system (1). It has been demonstrated that the strain having high proteolytic selectivity (14) are significantly more invasive than strains that produce little or no protease activity and similarly the infected animal has been thought to be associated with availability of antiproteases or antienzymatic factors in serum (15).

It is evident from several studies that extra cellular proteases play an important role in pathogenesis of pseudomonas infection (16). The intra corneal injection of Pseudomonas proteases produces gross corneal damage and structural alterations similar to those observed in authentic infection (17,18,19,20). These include the degeneration of corneal cells, the infiltration of polymorphonuclear leucocytes, loss of staining of stromal proteoglycans and the disposal of collagen fibrils (21). Many strains of Pseudomonas aeruginosa produce two distinct proteases termed as alkaline protease and elastase and the latter has been favored as major contributor to the pathogenicity (22,23). In addition to the proteolytic enzymes contributing to the pathogencity is another

important protein factor that help the microorganism in establishing host invasion is, the presence of protease inhibition in them (24). These protease inhibitors have been generally shown to be highly active against neutophil elastase (25). Several of such protease inhibitory substances have been studied extensively from a variety of pathogenic microorganisms (26). Since Neutrophil elastase can digest elastin (27) type III, type IV collagen (28) proteoglycans (29) and fibronectin (30) it has been implicated in mediation of connective tissue injury in many inflammatory disorders (31). It was therefore reasoned that the microbial elastase inhibitor might be a factor affecting the degree of tissue injury associated with pyogenic infection. It has been shown that crude extract of pneumococci can protect mice against acute lung injury induced by human neutrophil granule extract (32). Studies have suggested that different strain of pneumococci and other pyogenic bacteria may vary in their content of the elastase inhibitor and in their capacity to release the agent extracellularly. This variety might help to explain the different properties of these micro organisms to produce permanant lung injury as a sequel of pneumonia (33). In addition, different microorganisms may induce different levels of elastases, secretion from phagocytosing neutrophils; the more heavily encapsulated organisms (eg. Type III Pneumococci and Klebsiella) possibly are more stimulating as a result of being more difficult to engulf (25). Simultaneously, the thicker capsules surrounding these organisms could limit interactions between the inhibitor located within their membranes and the released protease. Finally, some bacteria produce and secrete proteases of their own and these enzymes are also potential participants in this process (33). One of the obvious possibilities of the protease / elastase inhibitor is that, it might help the bacteria to protect

associated with a number of biochemical functions which may be considered virulence factors. The virulence factors include surface proteins that promote colonization of host tissues, invasion that promote bacterial spread in tissues Eg: leukocidin, hyaluronidase etc., (40, 43, 44). There are studies which emphasize the need for looking for virulence factors as the cause of normal flora turning out opportunistic pathogens (45). There have been quite a good number of studies on protease inhibitors to demonstrate the role of these inhibitors as potential molecules with therapeutic uses as protease are shown to be involved in inflammatory responses by the host cells not only in traumatic situation but also in the infection. It is to certain extent clear that the Neutrophil elastase and the extraneous bacterial proteases all contribute to the tissue damage. Hence controlling of the activity of Neutrophil elastase and bacterial elastase might be important in spread of infection.

A sustained influx of inflammatory cells, mostly neutrophils into the air spaces is associated with lung injury from mechanical ventilation with high responding oxygen concentration and with early onset of neonatal or nosocomial bacterial infection. (46, 47, 48). These cells release elastase an enzyme capable of destroying various extracellular matrix components and proteins that play a role in host defense (49). In the lungs, several protease inhibitors including alpha 1 protease inhibitors and two acid resistant inhibitors namely mucus protease inhibitor or MPI, bronchial secretion inhibitor or BSI , antileukoprotease (ALP) and elastase specific inhibitor (ESI or Elafin) are very important for balancing elastase activity. Other inhibitors believed to be of minor importance include alpha1 antichymotrypsin and alpha 2 macroglobulin (50).

Bronchiectasis is characterized by a vicious cycle of infection and inflammation. Air way epithelium is damaged by infection, pollution etc. Bacteria colonize the damaged epithelium and cause neutrophil attraction by chemotactic stimuli (51). The activated neutrophil release, reactive oxygen radicals and proteolytic enzymes to destroy bacteria, which further damage the epithelium and protective protease inhibitors and in many cases the bacteria are not completely destroyed resulting in chronic inflammation (52, 53). Neutrophil elastase has been shown to cause pathological features in bronchiectasis including degradation of elastin (54) proteoglycans (55) and collagen (56). The Neutrophil elastase is inhibited by alpha 1 protease inhibitor, elafin inhibitor and secretory leukocyte inhibitor (57). Studies have shown that in the first stages of pneumonia there is a disturbed protease – anti protease balance which comes to normal if bacteria are cleared (58). Thus the studies have correlated the role of protease and anti protease in the infective processes.

Many different cell types were shown to produce elastase and elastase type proteases. Polymorphonuclear leukocytes neutrophils, smooth muscle cells, fibrinoblasts, blood platelets and endothelial cells (59). Human blood also contained elastase inhibitors (alpha 1 protein inhibitor and alpha 2 macroglobulin). Serum also contained elastolase type of peptidase insensitive to the circulatory alpha 1 protease inhibition

(60) Recent studies have suggested that low values of serum elastase activity and high levels of serum elastase inhibitors produced increased carotid plaque occurrence though the underlying mechanism which has not been elucidated (61).

Elastase activity has been recognized in various strains of Aspergillus fumigatus / flavus. The coexistence of elastase inhibitory activity in these organisms has also been demonstrated. The presence of the anti elastase substance has been suggested as a defense against host resistance mechanisms and could contribute to onset of mycosis (62). There are evidences suggestive of the ability of the fungal elastase inhibitor to inhibit leukocyte elastase which might be helpful to control inflammatory responses caused by leukocyte elastase (63). Studies on the production of extracellular elastase acid proteinase and phospholipase from various Aspergillus species are attributed to development of invasive aspergillous. However, statistical analysis has shown a correlation between existence of high phospholipase activity and this invasive disease (63).

It is evident from the above review of literature that there exists a very high functional relationship between the extra cellular proteolytic enzymes of pathogenic micro organism and development of diseases. Hence, to combat the devastating effect of the proteolytic enzymes on host cells, the host produce anti protease substances which have been characterized as peptides, proteins and new proteins. They have been shown to be active in exogenous and endogenous systems as defense substances for control of

proteolytic damage. The presence of these anti proteolytic substances particularly proteases is well established in micro organisms, plants and animals. They have also been shown to posses potent antibiotic activity against bacteria, fungi and even certain viruses. The rapid emergence of microbial pathogens that are resistant to currently available antibiotics has triggered considerable interest in isolation and investigation of mode of action of anti microbial peptides / proteins. The spectrum mode of action of these peptides / proteins could be varied. In this proposed study the focus is on anti elastase peptides / proteins substances of natural origin particularly from medicinal plants. The study includes the purifications of elastase from pathogenic bacteria and anti elastase substances. The study also focuses on the kinetics of the purified proteases and its interaction with anti proteases.

AIMS AND OBJECTIVES

1. The present study is aimed at a systematic screening of bacteria belonging to pathogenic and non pathogenic strains for total proteolytic activity (extra cellular) and fractionation of total proteolytic activity belonging to various classes of proteases namely serine, thiol proteases etc.
2. Elastase enzyme will be purified from Pathogenic and non pathogenic bacteria which show the highest activity of elastase. A comparison on their kinetics and other physiochemical properties will be carried out.
3. A comparison on HNE (Human Neutrophil elastase) and Bacterial elastase enzymes and their effects on known human protease inhibitors like alpha2 macroglobulin and alpha 1 antitrypsin etc. will be studied.

Studies will be extended if time frame permits to include the below mentioned objective.

Screening of the medicinal plants for anti elastase (bacterial origin) activity and purification of the factor from such source – The effects of the purified factor (anti elastase) on HNE and bacterial elastase will be studied extensively. Studies will be extrapolated for possible clinical / commercial application of the purified factor.

MATERIALS AND METHODS

The proposed studies utilize the bacteria belonging to both pathogenic and non pathogenic origin. The identification and characterization of the bacteria to be studied will be done by using appropriate methods explained in the literature. It is proposed to study the following pathogenic bacteria 1) Pseudomonas aeruginosa 2) Staphylococcus aureus 3) Arcanobacterium haemolyticum and Non pathogenic bacteria 1) Saphrophytic Staphylococcus 2) Micrococcus 3) Bacillus subtilis for screening of the total proteolytic activity and fractionation of the proteolytic activities. The Bacterial strains will be obtained in consultation with the department of Microbiology of our institution or adjoining institutions of this region. Culture techniques and preparation of the media for these organisms will be standardized as per the methods described in literature (64,65,66 & 67).

Estimation of total proteolytic activity in pathogenic and non pathogenic organisms will be done using Casein as substrate as per the procedure described by Sujatha.S and et.al. (68)

Estimation of Elastase will be done using succinyl tri-L-alanyl

p- nitroanilide (STANA) as substrate as per the procedure described by Bieth,J.,et.al (69)

Estimation of serine proteases and thiol proteases using enzyme specific substrates such as alpha-N-benzoyl DL-arginine p-nitroanilide (BAPNA) N-Acetyl L-tyrosine ethyl ester (ATEE), N-Benzoyl L-Arginine ethyl ester (BAEE) etc. as per the procedures described by Swaminathan.S et.al (70), Rao.N.R et.al (71) and A.C.Storer et.al (72)

The purification of the enzyme will be carried out using conventional protein purification methods involving salt fractionation, Ion exchange chromatography and affinity chromatography techniques. Attempts would be made on the use of Pseudo Ligand Affinity chromatography techniques for purification of the enzyme elastase. The homogeneity of the purified enzyme will be established by SDS - PAGE and by gel filtration chromatography.

Anti Proteolytic activity present in medicinal plant extracts will be assessed by systematically using suitable aliquots of the plant extracts in the enzyme catalysed reaction systems which would be measured against a control enzyme system without the plant extracts. For measurement of inhibition in total proteolytic activity Casein will be used as substrate. However, during assessment inhibition of various classes of proteases enzyme specific substrates will be used. One such medicinal plant which exhibits highest anti elastase activity will be purified to homogeneity

The Homogeneity of the anti enzymatic factors particularly the anti elastase will be assessed by using gel filtration, SDS PAGE and Iso Electric focusing.

Extensive studies will be conducted to assess mode of action of the anti enzymatic factors on purified elastase. Attempts will be made to establish the groups involved in the interaction through complex formation studies and modifications of R groups of the amino acids. The enzymes purified from pathogenic and non pathogenic strain will be subjected to extensive enzyme kinetic studies to establish similarities / dissimilarities between these enzymes.

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